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(54) Title: METHOD FOR IMMOBILISATION OF PROTEINS AND POLYELECTROLYTES ON SURFACES OF SOLIDS

(57) Abstract

Method of immobilization of proteins and polyelectrolytes on surfaces of solids in the form of films composed of molecular layers of proteins and polyelectrolytes arranged according to an eligible architecture characterised in that it includes the steps (a) the immobilisation of the protein or polyelectrolyte layer by the physical adsorption or a chemical bond, the step (b) the consecutive alternating adsorption of molecular layers of proteins and polyelectrolytes bearing opposite electrical charges, the step (c) the fixation of the resulting complex film by the chemical crosslinking and the step (d) the washing out of the uncrosslinked components.

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Description

Method for immobilisation of proteins and polyelectrolytes on surfaces of solids

Technical Field

The invention relates to a novel method of immobilisation of proteins and polyelectrolytes on surfaces of solids thereby forming surface films consisting of molecular layers of proteins and polyelectrolytes arranged according to an eligible architecture.

Background Art

Systems based on surface immobilised proteins are nowadays widely utilised, e.g. in medicine, pharmacology, and various biotechnologies.

The irreversible physical adsorption is currently used for immobilisation of proteins capable of a strong interaction with surfaces. The attachment is not usually very stable in the biological environment due to an exchange of the adsorbed protein for proteins in solution with a higher affinity for the surface. A strong physical interaction of the adsorbed protein with the surface often decreases its activity.

A most frequently used method of immobilisation is a chemical (covalent) binding between an active group of protein (e.g. amino, phenyl, and other nucleophilic groups) and an active group on the substrate surface. The surface binding via hydroxyl groups activated with bromcyanogen has been the most widely used method. The disadvantage is a low stability of the bond, an ion exchanging character of the activated surface and a toxicity of the reagents used. A more stable bonding via surface hydroxyl groups can be achieved by activation with chloroformates. Another type of active surfaces is represented by materials with reactive epoxide groups. The rather high pH (ranging from 9 to 13) necessary for the activation of the epoxide groups often damages the

reacting proteins. The methods mentioned above are used for the covalent bonding of proteins to hydrogel substrates made from hydrophilic polymers bearing corresponding reactive groups.

In practice it is often necessary or more desirable to immobilise proteins on surfaces of substrates which do not swell with water (e.g. porous particles and membranes, hollow fibres, tubing, prostheses and other solid subjects) and which do not posses corresponding groups for the covalent attachment. Active groups must be implanted to the surfaces of such materials by a surface modification. Surfaces of silicon materials are modified with chlorosilanes. Surfaces of inert polymers are modified using rather harsh methods (radiation grafting, plasma treatment, strong oxidation reagents) which may damage the product.

A common disadvantage of systems for the immobilisation of proteins on rigid surfaces is a substantial degree of deactivation of proteins caused by the physical interaction with the underlying surface and a presence of spots of a bare surface remaining between the attached protein molecules. A nonspecific adsorption of proteins from the surrounding solution as well as an activation of adverse reactions in the physiological environment may occur on the uncoated surface areas.

One way of a protein immobilisation on the inert rigid surfaces avoiding the above drawbacks is covering surfaces with a thick layer consisting of a crosslinked mixture of a hydrogel with the active protein. A uniform film can be mechanically deposited only on some objects (it cannot be deposited on, e.g., porous materials). Thick films, in general, adhere inadequately to substrates due to the contractions caused by changes in the degree of swelling. The protein immobilised inside the film is not active if the corresponding reagents (analytes) are too big to diffuse into the interior of the thick crosslinked film.

Disclosure of Invention

The object of the invention is a method for the immobi-

lisation of proteins and polyelectrolytes onto surfaces and formation of films consisting of molecular layers of proteins and polyelectrolytes which are arranged in an eligible architecture characterised in that (a) protein or polyelectrolyte is immobilised on the surface by the physical adsorption or by the covalent binding followed (b) by the consecutive alternating adsorption of the protein and polyelectrolyte under the conditions of formation of an ionic bond between the protein and the polyelectrolyte so that a film composed of alternating molecules of proteins and polyelectrolytes is formed and (c) the film is subsequently fixed by a chemical crosslinking between active groups of protein or between active groups of protein and polyelectrolyte; (d) the multilayer film of protein which does not contain another polylectrolyte can be formed by using polyelectrolyte without chemical groups reacting with crosslinker and by washing the polyelectrolyte from the crosslinked film with a solution in which the ionic bond between the protein and the polyelectrolyte is cancelled.

(a) The immobilisation of the first layer. The monomolecular layer of protein is obtained by contacting the surface with an aqueous solution of a globular protein, e.g., albumin, fibrinogen and globulin, which adsorbs irreversibly via hydrophobic interaction on materials with a high free interfacial energy between their surfaces and water, i.e., on surfaces of polymers like polyolefines, polystyrene, polyvinylchloride, fluorinated polymers, polyesters, silicones, polymethacrylates, polyamides, cellulose acetates, polyimides, polyacrylonitrile, polyurethanes, polycarbonates, epoxide and formaldehyde resins, natural rubber, or metals, semiconductors, ceramics, graphite, or which adsorbs via a combination of hydrophobic and ionic interactions on surfaces of hydrophilic solids like glass, quartz and mica. The physical adsorption is performed by a mere contact of an aqueous solution of the protein without any chemical activation of the surface or protein. The physical adsorption is not quite irreversible on neutral hydrogels consisting of

movable polymer chains like cuprophane, polyhydroxyalkyl-acrylates and methacrylates, polyacrylates, agarosa, sepharosa, polyacrylamide. This type of materials can be coated with the first protein layer by one of the known chemical methods. A coating mediated by an ionic interaction can be applied only if the ionic bond is stable during the subsequent formation of the multilayer film and if a covalent attachment of the protein to the surface can be formed during the crosslinking step. A polyelectrolyte can be immobilised by the physical adsorption via an ionic interaction with the opposite charged surface; a polyelectrolyte with reactive groups can be immobilised to an activated surface by a covalent bonding using the known methods. In both cases the protein which adsorbs onto the immobilised polyelectrolyte in the subsequent steps must form a covalent bond with the surface during the final crosslinking. For example, collagen, gelatine and crosslinked albumin or fibrinogen can be used as solid substrates.

b) The consecutive alternating adsorption of proteins and polyelectrolytes. Additional layers of the protein and polyelectrolyte are consecutively adsorbed by alternating contacts of the surface with the protein and polyelectrolyte solutions. By using for individual layers solutions of different proteins and/or polyelectrolytes films are prepared in which different proteins and/or polyelectrolyte alternate in a required sequences. The ionic strength and pH of solutions are chosen so that the protein and polyelectrolyte form ionic complexes. In a multilayer film a molecular layer of polyelectrolyte connects the neighbouring protein layers. Polysaccharides, polypeptides and synthetic polymers soluble in water, such as, e.g., polystyrene sulfonate, polyvinyl sulfonate, sulfonated cellulose, polymethacrylic acid and its copolymers, polyglutamates, polyvinylpyridine and polyionenes can be used as polyelectrolytes.

c) The crosslinking. Chemical crosslinks are formed by contacting the coated surface with a water solution of a crosslinker, e.g., glutaraldehyde, in which the ionic bonding between the protein and polyelectrolyte layers is maintained.

The crosslinker forms chemical bond between reactive groups of proteins (amino, phenyl and other nucleophilic groups). If the polyelectrolyte reacts with the crosslinker (e.g., via amino groups of some polysaccharides or synthetic polyelectrolytes), then the polyelectrolyte becomes an integral part of the covalent network.

d) The washing out of polyelectrolyte. Polyelectrolytes which do not react with the crosslinker can be washed out of the multilayer with a solution in which ionic interactions between the protein and polyelectrolyte are cancelled so that a covalently crosslinked film composed only of proteins is prepared.

The novel method, in comparison to known methods, is applicable to virtually any solid material which, with the exception of neutral hydrogels, need not to be chemically activated. The protein films are formed on ready made devices by a mere contact with the water solutions of the components to be deposited. Dimensions and shapes of the devices to be coated are unlimited. Uniform films are deposited on materials like foils, tubes, fibres, hollow fibres, powders, porous particles etc. Contrary to a monomolecular protein layer, the multilayer film with a defined number of layers is more resistant to the erosion in the biological environment. The thickness of the films is several orders of magnitude smaller and more uniform than that of films prepared by casting. For an easier storage and handling the films on solids can be dried and reswollen just before the use. Multilayer films composed of layers of different proteins can contain inner layers of an inactive protein (e.g. albumin) which forms an ideal transition from the solid surface to the outer layers of actively functioning protein. Such inner film minimises interfacial tension between the surface and water, minimises nonspecific adsorption and does not initiate adverse reactions of the organism. Active protein molecules immobilised on the film are better accessible to big reactants. The primary protein layers of e.g. albumin can serve as an anchor site for other proteins which do not adsorb directly on the surface. If the antigen or substrate for the immobilised

protein can migrate through an albumin film, the albumin membrane covering the active protein layer can protect it. Immobilised layers of different enzymes can be arranged into assemblies of mutually cooperating enzymes in which the product of one layer is a substrate for the enzyme in the neighbouring layer.

The invention is illustrated by the following examples of performance without, however, limiting the scope to them.

Examples

Example 1

The immobilisation of four molecular layers of serum albumin (SA) on the surface of the acetate cellulose membrane.

(1) The adsorption of SA from 0,1% SA in 0,05 M NaCl, HCl, pH 3,0 (NaCl solution) yields monomolecular layer of 0,24 µg SA/cm^2.

(2) The washing of the surface with the NaCl solution.

(3) The adsorption of sodium polystyrene sulfonate (PSS) from 0,1 % PSS in the NaCl solution.

(4) The washing of the surface with the NaCl solution.

(5) The adsorption of the second SA layer by repeating the step (1) yielded 0,44 µg SA/cm^2.

(6) The adsorption of the third and fourth SA molecular layer by repeating twice the sequence of the steps (2) to (5) yielded 0,42 µg SA/cm^2 0,45 µg SA/cm^2, respectively.

(7) The washing of the surface with the NaCl solution.

(8) The crosslinking of SA in the film by 0,5% glutaraldehyde in the NaCl solution.

(9) The washing of the surface with the NaCl solution.

(10) The washing of the surface with 0,1% SA in NaCl solution.

(11) The washing with water.

(12) The washing with phosphate buffer pH 7,4.

(13) The washing with water.

The washing removed from the crosslinked film residual PSS and glutaraldehyde and the final film contained 1,55 µg SA/cm^2.

Example 2

The immobilisation of three molecular layers of human fibrinogen (Fb) on Teflon.

(1) The adsorption of Fb from 0,025% Fb in 0,01 M citrate buffer, pH 4,0 (CB) yielded monomolecular layer of 0,49 µg Fb/cm^2.
(2) The washing of the surface with CB.
(3) The adsorption of sodium polystyrene sulfonate (PSS) from 0,1% PSS in CB.
(4) The washing of the surface with CB.
(5) The adsorption of the second Fb layer by repeating the step (1) yielded 1,37 µg Fb/cm^2.
(6) The adsorption of the third Fb molecular layer by repeating twice the sequence of the steps (2) to (5) yielded 0,42 µg SA/cm^2 and 0,45 µg SA/cm^2, respectively.
(7) The washing of the surface with CB.
(8) The crosslinking of Fb in the film by 0,5 % glutaraldehyde in CB.
(9) The washing of the surface with CB.
(10) The washing of the surface with 0,025 % Fb in CB.
(11) The washing with water.
(12) The washing with phosphate buffer pH 7,4.
(13) The washing with water.

The washing removed from the crosslinked film residual PSS and glutaraldehyde and the final film contained 3,43 µg Fb/cm^2.

Example 3

The immobilisation of three layers of gamma-immunoglobuline (IgG) on the surface of polyethylene (PE) coated with three layers of serum albumin (SA). (PE/SA-SA-SA-IgG-IgG-IgG)

(1) The adsorption of SA from 0,1 % SA in phosphate buffer pH 7,3 yields monomolecular layer of SA.

(2) The washing of the surface with water.
(3) The adsorption of sodium polystyrene sulfonate (PSS) from 0,1 % PSS in citrate buffer pH 4,2 (CB).
(4) The washing of the surface with CB.
(5) The adsorption of the second SA layer from 0,2 % SA in CB.
(6) The adsorption of the third SA molecular layer by repeating the sequence of the steps (4), (3), (5) and (4).The film contained 1 μg SA/cm^2 in the three molecular layers.
(7) The adsorption of IgG from 0,1 % IgG in CB yielded molecular layer of 0,52 μg IgG/cm^2.
(8) The adsorption of the second and third layer of IgG by repeating the sequence of the steps (2) to (4) and (6). The film of three IgG layers contained 2,4 μg IgG/cm^2.
(9) The washing of the surface with CB.
(10) The crosslinking of SA and IgG in the film by 0,5 % glutaraldehyde in CB.
(11) The washing of the surface with CB.
(12) The washing of the surface with 0,1 % IgG in water.
(13) The washing with water.
(14) The washing with phosphate buffer pH 7,4.
(15) The washing with water.

The washing removed from the crosslinked film residual PSS and glutaraldehyde.

Example 4

The immobilisation of three layers of urease (U) with a protective bilayer of albumin on the surface of gold (Au) coated with two layers of albumin. (Au/SA-SA-U-U-U-SA-SA)

(1) The adsorption of SA from 0,1% SA in citrate buffer pH 7,3 (CB) yields monomolecular layer of SA.
(2) The washing of the surface with CB.
(3) The adsorption of sodium polystyrene sulfonate (PSS) from 0,1 % PSS in CB.
(4) The washing of the surface with CB.
(5) The adsorption of the second SA and PSS layers by

repeating the sequence of the steps (1) to (4).

(6) The adsorption of U from 0,1 % U in CB.

(7) The washing of the surface with CB. The first urease layer contained 1 µg U/cm^2.

(8) The adsorption of PSS from 0,1 % PSS in CB.

(9) The washing of the surface with CB.

(10) The adsorption of the second and third U and PSS layers by repeating twice the sequence of the steps (6) to (9). The three molecular layers of urease contained 4,25 µg U/cm^2.

(11) The adsorption of two molecular layers of SA and PSS by the sequence of the steps (1) to (3), (4), (1) and (2). Two upper SA layers contained 0,9 µg SA/cm^2.

(12) The crosslinking of SA and U in the film by 0,5 % glutaraldehyde in CB.

(11) The washing of the surface with CB.

(12) The washing of the surface with 0,1 % SA in CB.

(13) The washing with water.

(14) The washing with phosphate buffer pH 7,4.

(15) The washing with water.

The washing removed from the crosslinked film residual PSS and glutaraldehyde.

Claims

1. Method of immobilisation of proteins and polyelectrolytes on surfaces of solids in the form of films composed of molecular layers of proteins and polyelectrolytes arranged according to an eligible architecture characterised in that it comprises the step (a) the immobilisation of the protein or polyelectrolyte layer by the physical adsorption or a chemical bond, followed by the step (b) the consecutive alternating adsorption of molecular layers of proteins and polyelectrolytes bearing opposite electrical charges, followed by the step (c) the fixation of the resulting complex film by the chemical crosslinking and followed by the step (d) the washing of the uncrosslinked components.

2. Method for immobilisation according to the claim 1 characterised in that the film composed only from protein components is received by using polyelectrolyte without chemical groups reacting with the crosslinker and by washing the polyelectrolyte out of the crosslinked film with a solution in which the ionic bond between the protein and/or proteins and the polyelectrolyte is cancelled.

INTERNATIONAL SEARCH REPORT

national Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K17/00 C12N11/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMISTRY LETTERS, no. 12, December 1994, TOKYO JP, pages 2323-2326, XP002008267 Y. LVOV ET AL.: "LAYER-BY-LAYER ASSEMBLY OF ALTERNATE PROTEIN/POLYION ULTRATHIN FILMS." see the whole document ---	1,2
Y	DE,A,41 41 302 (ZENTRALINSTITUT FÜR MOLEKULARBIOLOGIE) 17 June 1993 see column 2, line 53 - line 64; claims ---	1,2

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF THE CHEMICAL SOCIETY, CHEMICAL COMMUNICATIONS, no. 11, 7 June 1994, LETCHWORTH GB, pages 1297-1298, XP002008268 W. KONG ET AL.: "IMMOBILIZED BILAYER GLUCOSE ISOMERASE IN POROUS TRIMETHYLAMINE POLYSTYRENE BASED ON MOLECULAR DEPOSITION." see the whole document ---	1,2
A	DE,A,31 09 123 (FRAUNHOFER-GESELLSCHAFT ZUR FÖRDERUNG DER ANGEWANDTEN FORSCHUNG E.V.) 23 September 1982 see the whole document -----	1,2

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Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-4141302	17-06-93	NONE	
DE-A-3109123	23-09-82	NONE	